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Master's Thesis

# Sestrin2 interacts and regulates Orai1 calcium channels

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Department of Biological Sciences

Graduate School of UNIST

2020

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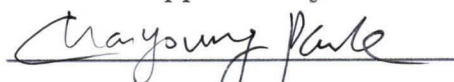
# Sestrin2 interacts and regulates Orai1 calcium channels

A thesis/dissertation  
submitted to the Graduate School of UNIST  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

Ki Hyun Jang

06. 16. 2020

Approved by



Advisor

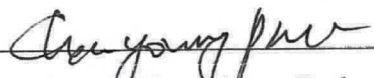
Chan Young Park

# Sestrin2 interacts and regulates Orai1 calcium channels


Ki Hyun Jang

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## Abstract

Store-operated  $\text{Ca}^{2+}$  entry (SOCE) is based on the interaction of endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  sensor stromal interaction molecule 1 (STIM1) with the Orai1  $\text{Ca}^{2+}$  channel which is located in plasma membrane. SOCE through Orai1 channels is induced by the store depletion in ER. Orai1 channels have unique regulation mechanism and its stable regulation is crucial for human health. To identify Orai1 activation and regulation mechanism further, I checked the proteins that bind with Orai1 through affinity purification coupled with mass spectrometry (MS). I identified approximately 3,000 proteins which bind with Orai1. I noticed sestrin2 (SESN2) among those proteins. Stress inducible protein SESN2 response to several stresses like DNA damage, hypoxia and oxidative stress. SESN2 is known to regulate cell growth, metabolism. In this research, I investigated the interaction between Orai1 and SESN2. I found that SOCE activation transcriptionally up-regulates SESN2 mRNA expression. Additionally, I confirmed that Orai1 binds and co-localizes with SESN2. Finally, To elucidate the functional role of SESN2 in SOCE activation, I examined the effects of SESN2 on SOCE. I confirmed that SESN2 activates SOCE by using Fura-2AM  $\text{Ca}^{2+}$  imaging and NFAT luciferase assay. These results indicate that SESN2 interacts and regulates Orai1 calcium channels.

## Key Words

SOCE, STIM1, Orai1, SESN2

## List of Abbreviations

**SOCE** Store Operated Calcium Entry

**SESN2** Sestrin2

**STIM1** Stromal interaction molecule 1

**PKC** Protein kinase C

**CRAC** Calcium release-activated channel

**ER** Endoplasmic reticulum

**MS** Mass spectrometry

**DMEM** Dulbecco's Modified Eagle's Medium

**FBS** fetal bovine serum

**AMPK** 5' adenosine monophosphate-activated protein kinase

**ROS** Reactive oxygen species

**mTORC1** mammalian targets of rapamycin complex

**TG** Thapsigargin

**PMA** Phorbol 12-myristate 13-acetate

**GAPDH** Glyceraldehyde 3-phosphate dehydrogenase



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## I. Introduction

Elevations of intracellular  $\text{Ca}^{2+}$  concentration regulate diverse biological functions from neurotransmitter release to proliferation, differentiation, gene expression, and apoptosis [1].  $\text{Ca}^{2+}$  controls these functions by interacting with numerous  $\text{Ca}^{2+}$  effector. This protein translates the signals to specific response of cells based on the dynamics of  $\text{Ca}^{2+}$  signals.  $\text{Ca}^{2+}$  signals play many roles, so the dysfunction of  $\text{Ca}^{2+}$  signal is related with diverse diseases and syndromes. The diseases are involved representatively cancer and immunodeficiency, autism and neurodegenerative diseases [2-6].

Store-operated channels (SOCs) are the major  $\text{Ca}^{2+}$  influx channel in non-excitabile cells [7]. SOCs induced sustaining  $\text{Ca}^{2+}$  elevation and triggered  $\text{Ca}^{2+}$  overload by both physiological or pharmacological processes [8,9]. The components and regulation mechanism of SOCs have been diligently discussed since the discovery of SOCE. The calcium release-activated channels (CRAC) is the most well-established SOCs, which is specialized plasma membrane highly selective  $\text{Ca}^{2+}$  channel [10]. STIM family and Orai calcium channels are two molecular components of CRAC, which mediate SOCE after calcium store depletion: The STIM proteins are the calcium sensors in the ER which are located in the membrane of the ER [11,12]. The Orai channels are well established for major function as activating SOCE by binding with STIM proteins [13,14]. The identification of the STIM proteins and the Orai calcium channels has allowed deep comprehension of the unique mechanism of SOCE. But, SOCE was not studied fully yet, so many researches have intensely studied the regulation mechanism of Orai calcium channels recent years.

In this research, I confirmed that approximately 3,000 proteins are binding with Orai1 calcium channels by using mass spectrometry. I took notice of SESN2 among them. SESN2 belong to the SESN family which is highly conserved proteins. There are 3 members in this family. All proteins do work for attenuating aging and regulates metabolism. The SESN2 is mostly studied in now. [15-17]. Stress inducible protein SESN2 is well known to regulate cell growth, metabolism, and play a role as the positive regulator of autophagy [18,19]. This protein response to several stresses like oxidative stress, hypoxia, and DNA damage [15,20]. There are two major biological functions among the various functions of SESN2. First, SESN2 can decrease the accumulation of reactive oxygen species (ROS) as antioxidants [21]. Second, SESN2 play as an inhibitor of mammalian targets of rapamycin complex1 (mTORC1) through the activation of AMPK [22,23]. Through these functions, SESN2 alleviates several age-related metabolic disorders, including muscle degeneration, cardiac dysfunction, mitochondrial dysfunction, glucose intolerance and insulin resistance [24-27]. Understanding the role of SESN2 in cell metabolism and age-related diseases is vital to develop new therapeutic method for age-related

diseases, so the activity of SESN2 in biological functions should be analyzed and discussed. In this study, I aimed to investigate the interaction between Orai1 and SESN2 and determine how Orai1 and SESN2 affect each other.

## II. Materials and Methods

### 2.1. Reagents

TG (Santa Cruz), Fura-2AM (Invitrogen), PMA (Santa Cruz), RiboEx (GeneAll), cDNA synthesis Kit (TOYOBO), GFP target antibody (MBL), FLAG M2 antibody (Sigma) were purchased from the indicated vendor.

### 2.2. Cell culture

HEK293 and HEK293T cells were used in these all experiments. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% or 10% fetal bovine serum (FBS). All cells were maintained at 37 °C in 5% CO<sub>2</sub> humidified atmosphere.

### 2.3. RT-PCR

RiboEx was used for isolating mRNA in cells. RNA reverse transcription was done by utilizing cDNA synthesis kit and oligo primers for making cDNA. Gel electrophoresis was done for identifying the PCR products. To compare expression of corresponding genes by RT-PCR, the indicated primers were used for PCR. The PCR was performed by C1000 Touch thermal Cycler.

#### Primers.

##### SESN1

Forward primer: '5-ggatccatgcgcttgccgccgccgcg-3'

Reverse primer: '5-tcaggtcatatagcgggtaatg-3'

##### SESN2

Forward primer: '5-ggatccatgatcgtggcggactccgag-3'

Reverse primer: '5-tcaggtcatgtagcgggtgatggcacg-3'

##### SESN3

Forward primer: '5-ggatccatgaaccggggcgccggcag-3'

Reverse primer: '5-tcaggtcaaagccgagttatg-3'

#### GAPDH

Forward primer: 5'-ctgaacgggaagctcactggcatg-3'

Reverse primer: 5'-aggtccaccacctgttgctgtagc-3'

### **2.4. Western blot and Immunoprecipitation**

Indicated products (FLAG-tagged-STIM1, FLAG-tagged-Orai1, FLAG-tagged-SESN2, YFP-SESN2, YFP-Orai1 NT, YFP-Orai1 23L, YFP-Orai1 CT) were expressed in cells for each condition. The cells transiently expressing indicated products were washed with phosphate-buffered saline. The cells were lysed by lysis buffer which is composed of Tris-HCl, NaCl. Lysates were centrifuged at 12,000 rpm for 5-15 min. The FLAG M2 beads were incubated with the supernatant at 4°C for overnight. The lysates and immunoprecipitated samples were run on an SDS/PAGE system. The gel was analyzed by chemiluminescence.

### **2.5. Confocal Microscopy**

The cells transiently expressing indicated products (Cherry-Orai1, GFP-SESN2 and split GFP system products) were detected and acquired by LSM780 confocal microscope. Cherry excitation was done at 595 nm and emission was detected at 600-650 nm. GFP excitation was done at 488 nm and emission was detected at 515-565 nm. Images were collected and analyzed by utilizing zen software. All experiments were performed at 25°C.

### **2.6. TIRF Microscopy**

The cells transiently expressing indicated products (Cherry-Orai1 and GFP-SESN2) were detected and acquired by ELYRA P.1 microscope. Cherry excitation was done at 595 nm and emission was detected at 600-650 nm. GFP excitation was done at 488nm and emission was detected at 515-565 nm. Images were collected and analyzed by utilizing zen software. All experiments were performed at 25°C.

## **2.7. NFAT Luciferase reporter assays**

The HEK293T cells expressing indicated products (YFP-SESN2 and empty vector) were analyzed by NFAT luciferase reporter assays. The NFAT luciferase reporter was transfected in cells with Renilla luciferase. The Renilla luciferase was transfected for control of transfection efficiency and cell number. The cells were treated by PMA and TG for 6h. The automated luminator was used for measuring the luciferase activity in samples. The relative luciferase activity was calculated as ratio of firefly to Renilla luciferase activity.

## **2.8. Intracellular $\text{Ca}^{2+}$ Imaging**

Indicated products (YFP-SESN2 and YFP) were expressed in HEK293 cells. The cells intracellular  $\text{Ca}^{2+}$  concentration was detected and analyzed by ratiometric Fura-2AM  $\text{Ca}^{2+}$  imaging.  $\text{Ca}^{2+}$  imaging was done at 340nm and 380nm by utilizing IDX83 microscope. Images were collected by MetaMorph software (Molecular Devices) and analyzed by Igor Pro (WaveMetrics) and Prism5.

## **2.9. Mass spectrometry**

FLAG-tagged Orai1 was expressed in HEK293 cells. Protein complex of a target protein of Orai1 was purified based on affinity tag, FLAG. The purified protein complex was digested by protease for mass spectrometry analysis. The protein identification was confirmed by utilizing algorithm based on the sequence database. The mass spectrometry data was analyzed by scaffold4 software.

## **2.10. Split-GFP complementation assays**

The plasmids GFP11-SESN2, GFP1-10-Orai1, and GFP10-Orai1 were designed from pM159-GFP10, pM162a-GFP1-9, pM214-GFP11, and pM423-GFP1-10. All original plasmids were received from Hajin Kim laboratory in UNIST. The indicated products were expressed in HEK293 cells for each condition. All images acquired by LSM780 confocal microscope. All experiments were performed at 25°C.

## 2.11. Statistics

Prism 5 was used for statistics in this research. Two-tailed t test was utilized for testing the pairwise differences. Statistically important or significant data was considered at P value  $< 0.05$ , indicated as \*P. The \*\*\*P means P value  $< 0.001$ .

### III. Results

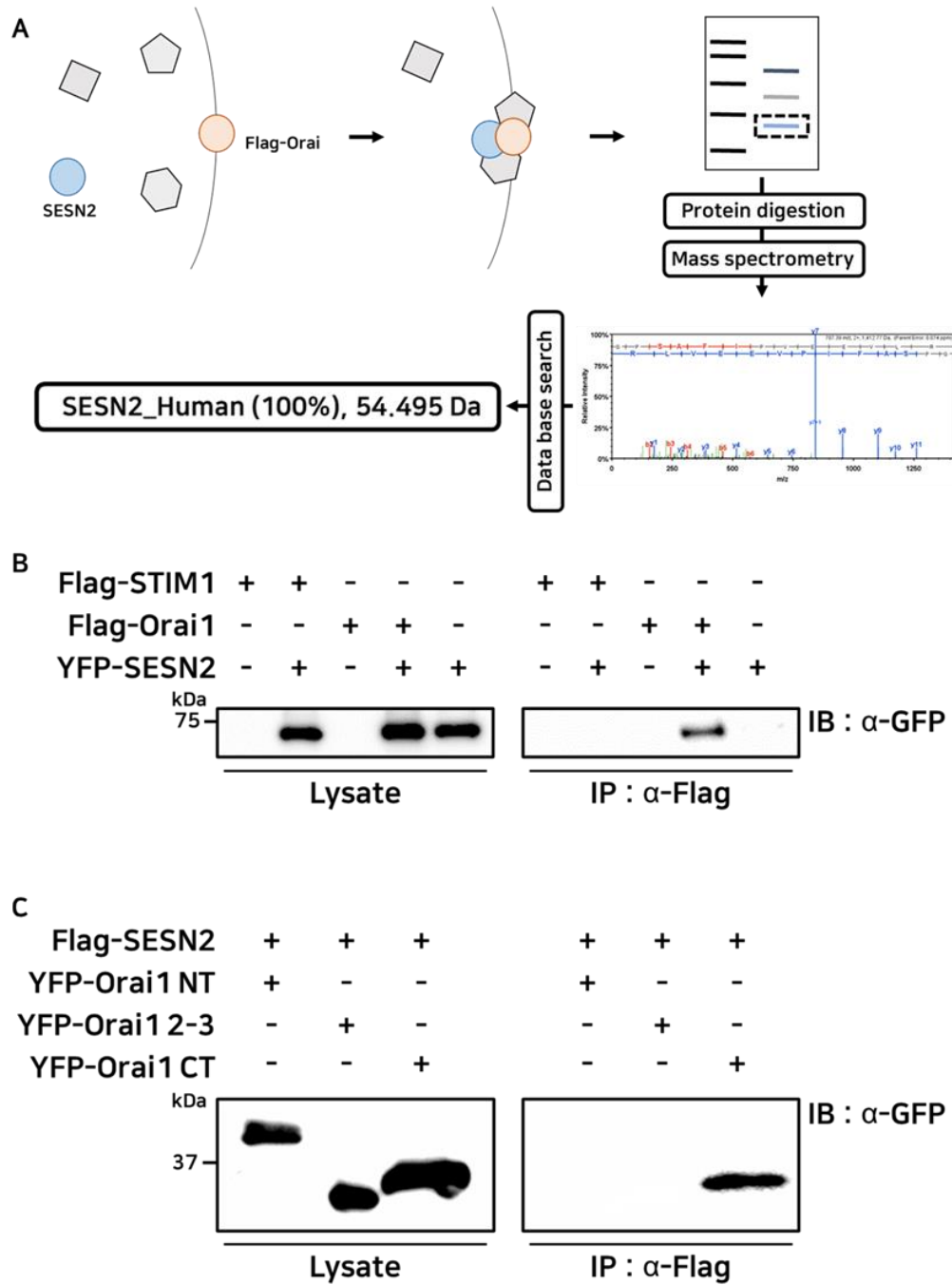
#### 3.1. SESN2 binds with Orai1 C-terminus

To identify unknown regulation mechanism and function of Orai1, I initially found interacting proteins of the Orai1 by using affinity purification coupled with MS. FLAG-tagged-Orai1 was expressed in HEK293 cells, and then I purified a protein complex of a target protein of Orai1 based on affinity tag, FLAG. The purified protein complex was digested by protease for MS analysis. The protein identification was confirmed by utilizing algorithm based on the sequence database. I identified that approximately 3,000 proteins which bind with Orai1. Additionally, I found interacting proteins of STIM1 by same method. I identified that approximately 200 proteins which bind STIM1. To specifically study the proteins which I confirmed that Orai1 specifically have approximately 900 candidates of interacting proteins except for interacting proteins that binds with Orai1 and STIM1 commonly. I analyzed the list of interacting proteins of Orai1 and I selected the SESN2 for studying the Orai1 regulation mechanism and specific biological function which can be regulated by those two proteins.

To identify whether SESN2 binds only to Orai1 but not to STIM1, I labeled the STIM1, Orai1 with FLAG and SESN2 with YFP tags. I subsequently expressed the indicated constructs in cells. The YFP-SESN2 only showed coimmunoprecipitation with FLAG-tagged Orai1. But YFP-SESN2 not showed coimmunoprecipitation with FLAG-tagged STIM1. This result means that SESN2 only binds with Orai1 but not with STIM1 (Figure 1B). I additionally identified that the SESN2 target domains within Orai1 by using immunoprecipitation. I transiently co-expressed YFP-tagged-three cytosolic domains, YFP-Orai1 NT (N-terminus), YFP-Orai1 23L (23 loop), and YFP-Orai1 CT (C-terminus) in cells. The FLAG-tagged-SESN2 only showed coimmunoprecipitation with YFP-Orai1 CT. But YFP-SESN2 did not show coimmunoprecipitation with YFP-Orai1 NT and YFP-Orai1 23L. This result means that Orai1 CT is the target domain of SESN2 (Figure 1C). These results implied that SESN2 might be a potent regulator of Orai1 channels through binding at the Orai1 CT.



**Figure 1.**



**Figure 1. SESN2 binds with Orai1 C-terminus.**

(A) Schematic representation of mass spectrometry.

(B) Indicated products were expressed in HEK293T cells. SESN2 binds with Orai1 but not STIM1.

(C) Indicated products were expressed in HEK293T cells. SESN2 binds with Orai1 C-terminus.

### 3.2. SESN2 co-localizes with Orai1 in plasma membrane

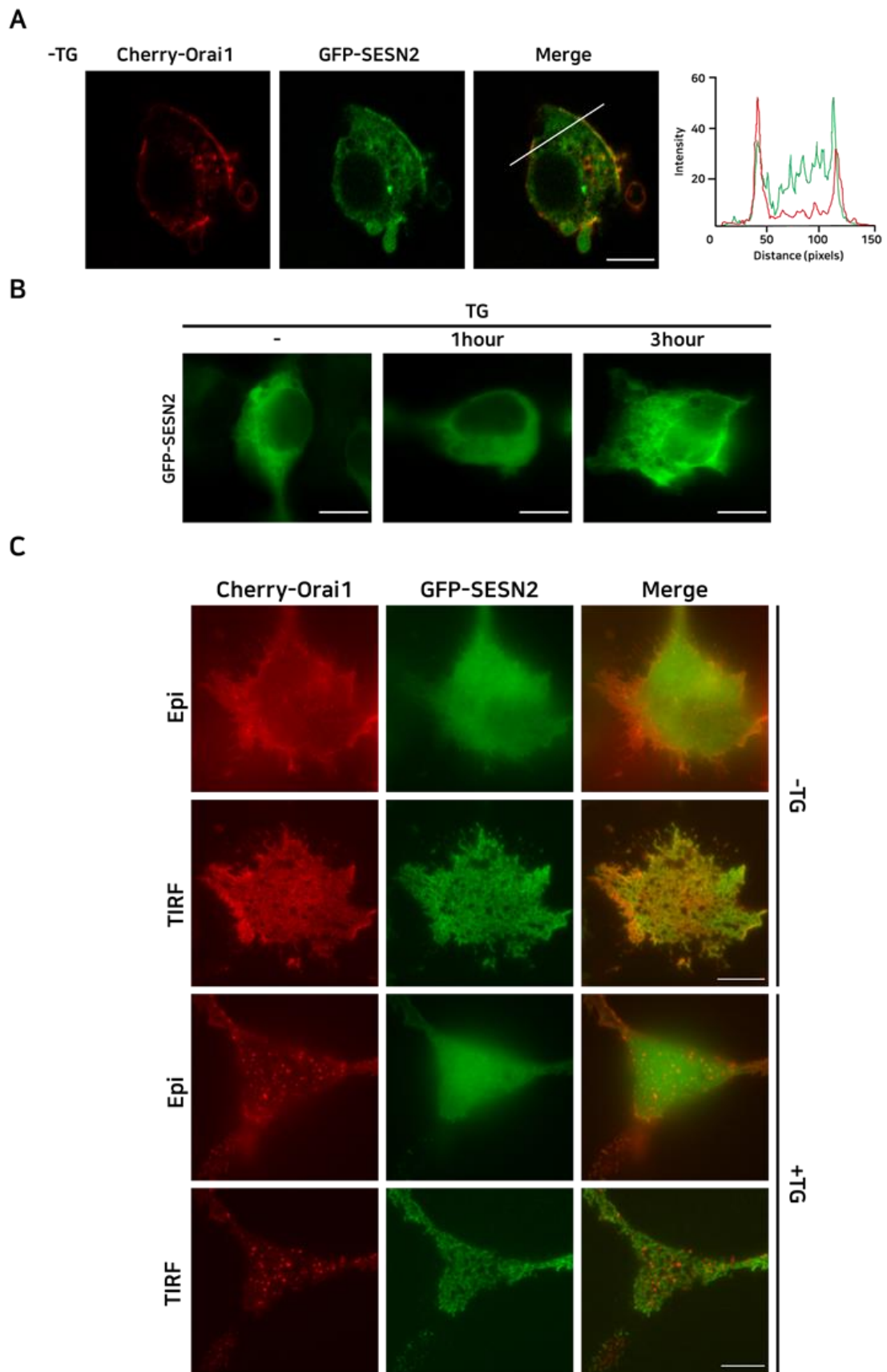
I initially confirmed that SESN2 binds to Orai1. Next, I wonder that SESN2 co-localizes with Orai1. To confirm the co-localization between SESN2 with Orai1, I checked localization of these two proteins by using several co-localization experiments. I labeled the Orai1 with Cherry tag and SESN2 with GFP tag. I co-expressed Cherry-Orai1 with GFP-SESN2 in HEK293 cells. I next analyzed the localization of Orai1 channels and SESN2 protein. The fluorescence of GFP-SESN2 was evenly distributed in the cytoplasm as well as plasma membrane in HEK293 cells. The fluorescence of Cherry-Orai1 was evenly distributed in the plasma membrane. I analyzed the co-localization between SESN2 and Orai1 in plasma membrane by using line scan analysis. The fluorescence of Cherry (red) and GFP (green) showed high intensity at both ends. This means that SESN2 co-localize with Orai1 in plasma membrane. Although the localization of SESN2 is evenly distributed in entire position of cells, it is clear that SESN2 localizes in plasma membrane and co-localize with Orai1 (Figure 2A).

I wonder if the localization of SESN2 is changed by SOCE activation. To address this issue, I expressed only GFP-SESN2 in HEK293 cells. I analyzed the localization of SESN2 in resting cells and in cells treated with thapsigargin (TG) to deplete ER calcium and activates SOCE for 0, 1, and 3 hours. The fluorescence pattern of GFP-SESN2 was not significantly changed in cells treated with TG for 1 hour. But the fluorescence of GFP-SESN2 localizes in plasma membrane of cell treated with TG for 3 hours (Figure 2B). This result suggests that localization of SESN2 is changed by SOCE activation.

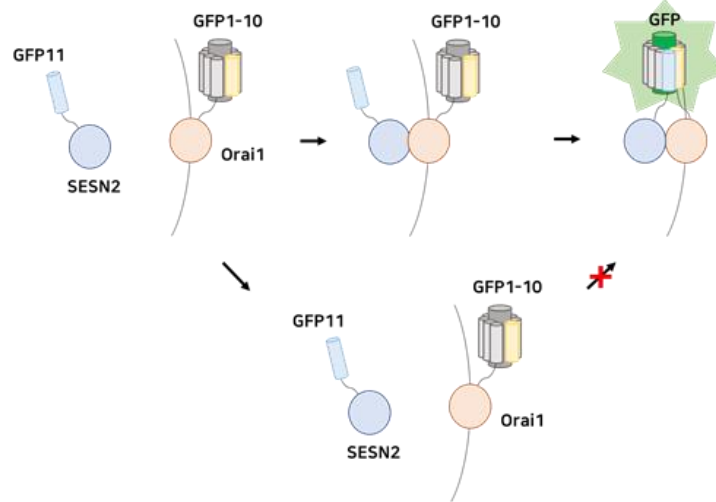
Next I specifically checked localization of Orai1 and SESN2 in plasma membrane by using TIRF imaging. I co-expressed Cherry-Orai1 with GFP-SESN2 in HEK293 cells. I next analyzed the co-localization of Orai1 and SESN2 in resting cells and in cells treated with TG to deplete ER calcium and activates SOCE. The fluorescence of Cherry-Orai1 is evenly distributed in plasma membrane of resting cells. The fluorescence of GFP-SESN2 also is evenly distributed in plasma membrane of resting cells. Two proteins occupied the entire position of plasma membrane in resting cells. It means SESN2 localizes in plasma membrane and co-localize with Orai1. Next, I analyzed the localization of SESN2 and Orai1 in cells treated with TG. Cherry-Orai1 formed puncta after ER calcium depletion. It means TG worked well. But GFP-SESN2 did not form puncta in cells. Even though some Cherry-Orai1 puncta co-localize with GFP-SESN2. Most of the Orai1 puncta locations are different from fluorescence pattern of SESN2. It is not clear that SESN2 co-localize with Orai1 in cells treated with TG (Figure 3C). These results indicated that SESN2 occupied same localization with Orai1 in plasma membrane of resting cells. But the intensity of co-localization decreased in cells after ER calcium depletion and followed SOCE activation.

Even though I confirmed that SESN2 co-localized with Orai1 in plasma membrane of resting cells, It is hard to decide co-localization those two proteins in cells after ER calcium depletion. To address this issue, I performed co-localization experiments by using Split-GFP complementation assays. There are two Split-GFP complementation assays including bipartite and tripartite split GFP systems. GFP1-10 is fused to Orai1 and GFP11 is fused to SESN2 in case of bipartite split GFP-complementation assay. If two protein interact in plasma membrane, full-length GFP is formed and GFP signal is shown in plasma membrane of cells (Figure 2D). GFP10 and GFP11 are respectively fused to Orai1 and SESN2 in case of tripartite split GFP complementation assay and the fragment GFP1-9 is needed for forming full-length GFP. If two proteins interact, full-length GFP is formed and GFP signal is shown in plasma membrane of cells (Figure 2E). First, I respectively expressed GFP1-10-Orai1, GFP10-Orai1, GFP11-SESN2 and GFP1-9 in HEK293 cells and then I confirmed that GFP fluorescence did not occur in each condition. I co-expressed GFP1-10-Orai1 with GFP11-SESN2 in HEK293 cells and I confirmed that GFP fluorescence was observed in bipartite split GFP assay. Likewise, I co-expressed GFP10-Orai1 and GFP11-SESN2 with GFP1-9 in HEK293 cells and I confirmed that GFP fluorescence was observed in tripartite split GFP assay (Figure 2F). These results indicate that the Orai1 interact with SESN2. I next checked the interaction of Orai1 and SESN2 in resting cells and in cells treated with TG by using bipartite and tripartite split GFP assays. The fluorescence of GFP was distributed evenly in plasma membrane in resting cells. Some puncta shaped fluorescence was shown after store depletion by TG (Figure 2G). These result means that the SESN2 interacts with Orai1 in plasma membrane of resting cells and cells after ER calcium depletion.

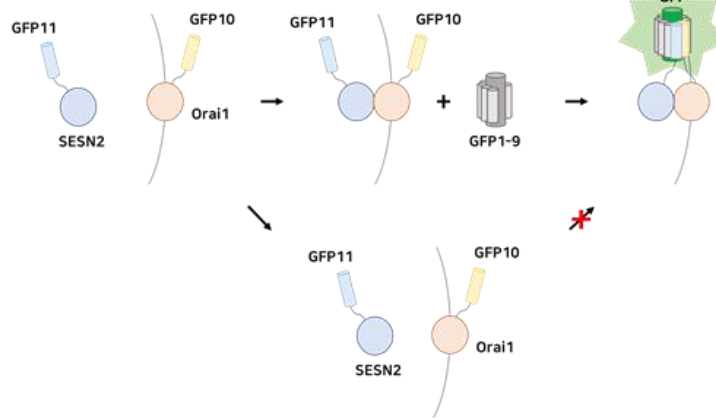
Figure 2.



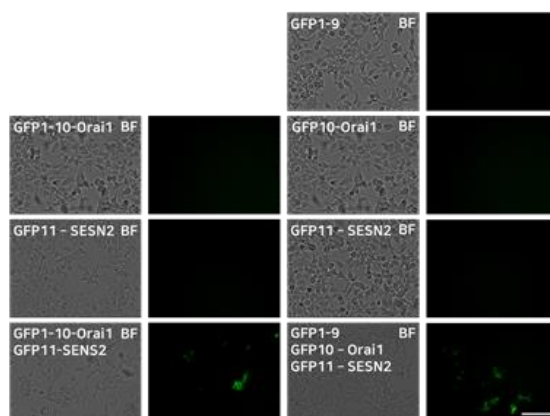
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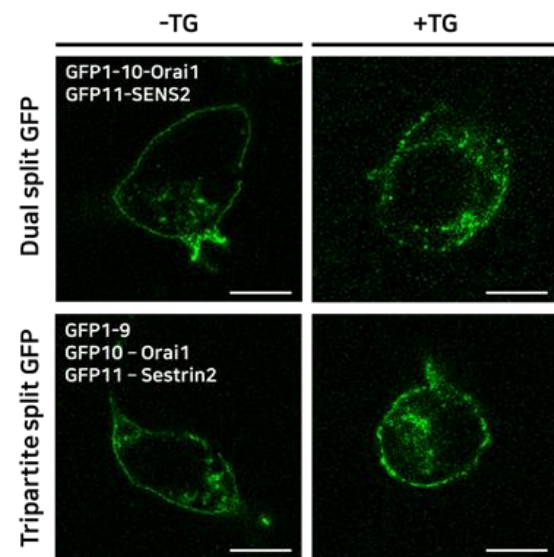
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**G**



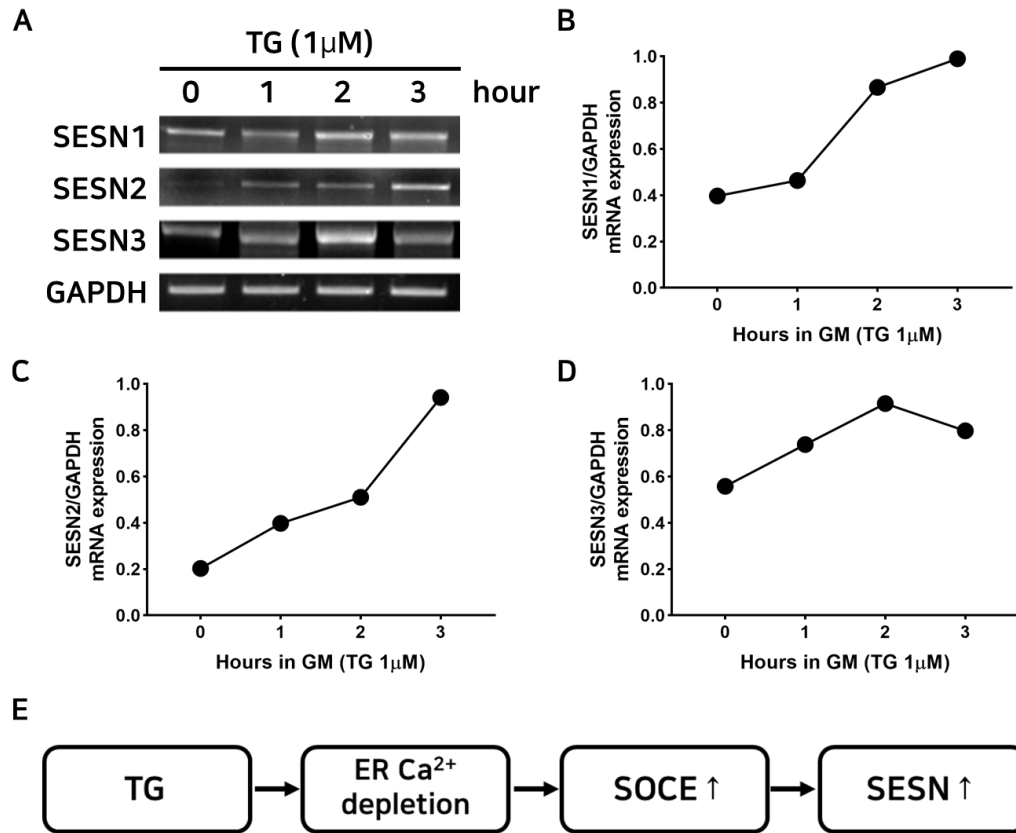
**Figure 2. SESN2 co-localizes with Orai1.**

- (A) Imaging showing co-localization of Orai1 (red) and SESN2 (green) in HEK293 cells.
- (B) Imaging showing localization of SESN2 in resting cells and in cells treated with TG for 0, 1 and 3 hours.
- (C) Imaging showing co-localization of Orai1 (red) and SESN2 (green) in resting cells and in cells treated with TG.
- (D) Schematic representation of bipartite split GFP assay
- (E) Schematic representation of tripartite split GFP assay.
- (F) Imaging showing co-localization between Orai1 and SESN2 in cells by using split GFP assays.
- (G) Imaging showing co-localization of Orai1 and SESN2 in resting cells and in cells treated with TG by using split GFP assays.

### 3.3. Expression of SESN genes is up-regulated by SOCE activation

To study the regulation mechanism of Orai1, I analyzed interacting protein of Orai1 by MS. I focused on the SESN2 those candidates of interacting protein of Orai1. The SESN2 have additional conserved proteins, SESN1 and SESN3. SESN family responses to several stress conditions and is transcriptionally regulated by them. I wonder that the expression of SESN family is regulated by SOCE activation condition. To check the mRNA the expression of SESN genes, I used RT-PCR assays. I confirmed the expression of SESN family genes in resting cells and treated with TG to deplete ER calcium and activates SOCE for 1, 2, and 3 hours. The results indicated that the expression of all SESN family genes are up-regulated by SOCE activation (Figure 3A-D). Even though SESN2 is only candidate of interacting proteins of Orai1 based on MS data, the expression of SESN1 and SESN3 also are up-regulated by the SOCE activation. Notably, the expression of SESN2 is significantly increased by TG. It means that even though all SESN family genes have possibility of potent regulator of SOCE, SESN2 might be the most important regulator of Orai1. Consequently, these results indicated that the ER  $\text{Ca}^{2+}$  store depletion and followed SOCE activation transcriptionally up-regulates SESN family genes induction (Figure 3E).

**Figure 3.**



**Figure 3. Expression of SESN family genes is up-regulated by SOCE activation**

(A) mRNA expression of SESN family genes after treating TG.

(B) RT-PCR analysis of SESN family genes expression, SESN1 is normalized by GAPDH.

(C) RT-PCR analysis of SESN family genes expression, SESN2 is normalized by GAPDH.

(D) RT-PCR analysis of SESN family genes expression, SESN3 is normalized by GAPDH.

(E) Diagram of Figure 3

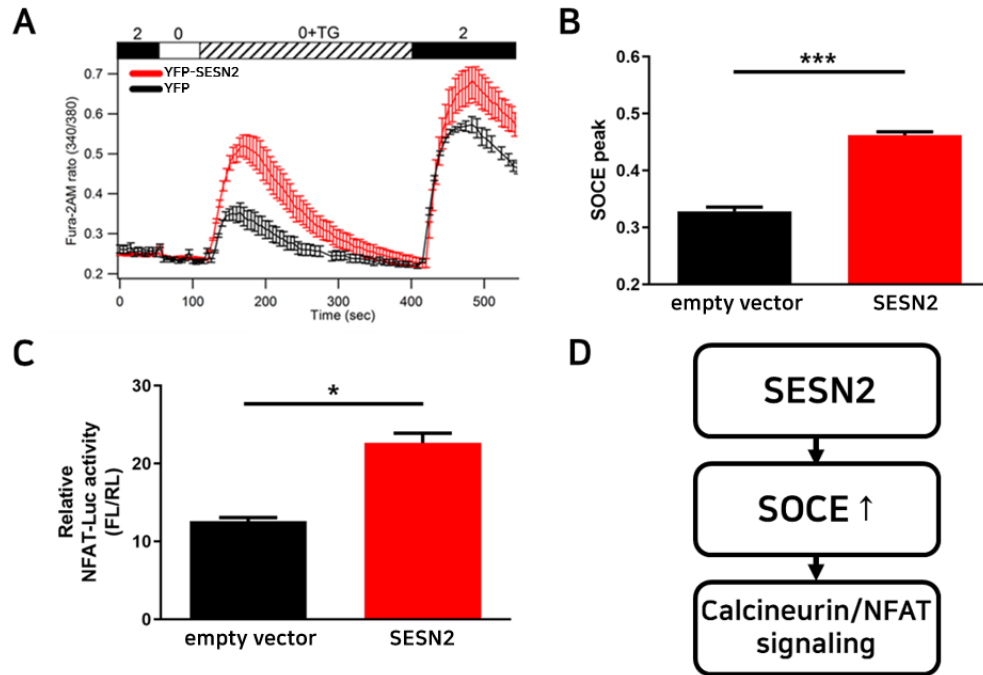


### 3.4. SESN2 up-regulates SOCE

I wonder that SESN2 can be the potent regulator of Orai1. To address this issue, I checked whether SESN2 activates or inhibits SOCE by Fura-2AM calcium imaging. I expressed YFP-SESN2 in HEK293 cells and treated with TG for ER calcium depletion. Next I measured intracellular calcium concentration after adding extracellular calcium. The increased intracellular calcium concentration reflects the extent of SOCE activation which is regulated by endogenous STIMs and OraIs. The cells transiently expressing SESN2 showed higher SOCE peak compare to control. It means that SESN2 up-regulates SOCE and it might be positive regulator of Orai1. (Figure 4A and B).

I confirmed that SESN2 activates SOCE, so I wonder if SESN2 can activate downstream calcium signaling which is regulated by SOCE. To address this issue, I identified whether SESN2 effects on the nuclear factor of activated T cells (NFAT) signaling pathway or not. I expressed NFAT luciferase reporter and Renilla luciferase in HEK293T cells. Transcription of NFAT needs increased concentration of intracellular calcium and protein kinase C (PKC) activation. To activate PKC, phorbol 12-myristate 13-acetate (PMA) was added with TG in HEK293T cells. The relative NFAT luciferase activity measured by dividing the Renilla to the firefly luciferase activity. The cells expressing SESN2 showed higher relative luciferase activity compare to control (Figure 4C). It means that SESN2 up-regulates SOCE and followed SOCE activates down stream calcium signaling pathway (Figure 4D).

**Figure 4.**



**Figure 4. SESN2 activates SOCE.**

(A) YFP-SESN2 (red) and YFP (black) are expressed in HEK293 cells respectively. Representative Fura-2AM calcium imaging in HEK293 cells.

(B) SOCE peak is measured in bar graph based on figure 4A.

(C) Empty vector and SESN2 expressed in HEK293T cells respectively. The relative NFAT luciferase activity is measured in cells treated with indicated products.

(D) Diagram of Figure 4

## IV. Discussion

Protein-protein interaction form protein complex that consists of two or more proteins which are connected together. The proteins reveal transient or stable and direct/indirect interaction within the protein complex. Most of the cellular processes and molecular functions are regulated by protein complexes. The research of protein complex is critical to develop our understanding on proteins functional roles in physiological and pathological conditions.

In this research, to study the regulation mechanism of Orai calcium channels, I identified approximately 3,000 proteins which bind with Orai1 calcium channels. Additionally, I found interacting proteins of STIM1. I confirmed that Orai1 specifically have approximately 900 candidates of interacting proteins except for interacting proteins that bind with Orai1 and STIM1 commonly. I took notice of SESN2 among those proteins. SESN2 belongs to the SESNs family which is highly conserved proteins. The SESN2 is mostly studied in now. SESN2 is well known to regulate cell growth, metabolism, and act as a positive regulator of autophagy [18-19]. and response to several stresses including oxidative stress, hypoxia and DNA damage [15,20]. I studied interaction between Orai1 calcium channels and SESN2 in this research. I found that SESN2 binds with Orai1 by MS and immunoprecipitation experiments. I confirmed that Orai1 co-localize with SESN2 by several co-localization experiments. I also identified that Orai1 and SESN2 intermittently bound and detached with each other in a short time by live imaging (data not shown). Additionally, I found that SOCE activation promote SESN2 mRNA expression level. To elucidate the functional role of the SESN2, I examined the effects of SESN2 on SOCE by NFAT luciferase assay and Fura-2 calcium imaging. I confirmed that transiently expressing SESN2 activates SOCE. These results indicate that SESN2 interacts and regulates with Orai1 calcium channels.

In the further study, I need an additional experiment whether SESN2 binds directly or indirectly with Orai1. In the Figure 1C, SESN2 binds to the Orai1 C-terminus. I have to further study how Orai1 C-terminus target protein modulates Orai1 calcium channels. Then I can guess how SESN2 regulates Orai1 by studying how other proteins control Orai1. In the Figure 2C, the proportion of co-localization between SESN2 and Orai1 low in cells treated with TG. It means that SESN2 do not need to binds with Orai1 after SOCE activation. I need to further study why SESN2 detach from Orai1 after SOCE activation. In the Figure 3, I found that mRNA expression of SESN2 was up-regulated by TG for ER calcium depletion and followed SOCE activation. I suggested that SOCE activation might regulates expression of SESN2, but TG is known to evoke an increase of ROS [28-29]. There is possibility that TG induced ROS up-regulates expression of SESN2. I need to check whether other SOCE activators up-regulate expression of SESN2. If other SOCE activators do not up-regulates expression of SESN2,

Figure 3 just showed that TG induced ROS up-regulates expression of SESN2. In the Figure 4, cells transiently expressing SESN2 showed high SOCE peak and activated SOCE downstream calcium signaling pathway, which means that somehow SESN2 interacts and regulates Orai1 calcium channels for SOCE activation.

In summary, my data indicated the link between Orai1 and SESN2. To study the regulation mechanism of Orai1 calcium channels, I found candidates that binds with Orai1 and I selected SESN2 among them. I identified that Orai1 and SESN2 are closely related and have positive feedback in activation. Since Orai1 and SESN2 play a vital role in the life and death of cells, The research is needed to identify how the interaction between the two proteins affects to specific phenomenon that occur within cells. Specifically, for example, since both proteins play a role in promoting autophagy [30]. It's very interesting to identify how the interaction between them is involved in regulating autophagy. Basically, SESN2 plays an important role in maintaining cell life. Thus, research of interaction between SESN2 and Orai1 will provide the potential therapeutic approach for the diverse SOCE deficiency related diseases and aging related diseases.

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